Ecotoxicology and Environmental Safety 98 (2013) 74–81

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Avermectin induced liver injury in pigeon: Mechanisms of apoptosis and oxidative stress

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ARTICLE INFO

Article history:
Received 5 May 2013
Received in revised form
8 September 2013
Accepted 12 September 2013
Available online 15 October 2013

Keywords:
Avermectin
Pigeon
Liver
Apoptosis
Oxidative stress

ABSTRACT

Extensive use of avermectin (AVM) can result in environment pollution, and it is important to evaluate the potential impact this antibiotic has on ecological systems. Few published literatures have discussed the liver injury mechanisms induced by AVM on birds. In this study, pigeons were exposed to feed containing AVM (0, 20, 40 and 60 mg/kg diet) for 30, 60, 90 days respectively. The results showed that AVM increased the number of apoptosis and the expression level of caspase-3, 8, fas mRNA in the liver of pigeons. Ultrastructural alterations, including mitochondrial damage and chromatin aggregation, become severe with increase exposure dose. Exposure to AVM induced significant changes in antioxidant enzyme {superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)} activities and malondialdehyde (MDA) content, augmented protein carbonyl (PCO) content and DNA-protein crosslink (DPC) coefficient, in a concentration-dependent manner in the liver of pigeons. Our results show that AVM has toxic effect in pigeon liver, and the mechanism of injury caused by AVM is closely related to apoptosis and oxidative stress.

1. Introduction

Compared to well-studied physiological and toxicological effects of AVM in insects, AVM metabolism in pigeon is still an unexplored field. Since birds belong to higher trophic level, they may play a key role in AVM circulation in the ecosystem. AVM is a widely used agent in agriculture and veterinary medicine in prevention of parasitic diseases by affecting the nervous system and paralyzing insects (Floate et al., 2005; Kövecses and Marcogliese, 2005). AVM undergo little metabolism in animals and up to 80–98% of the initial administered dose can be found in feces after metabolic process (Sun et al., 2005). Some literature reveal the target invertebrates, which were exposed to AVM with a similar mode of action-ivermectin, shows signs of ataxia and paralysis in their behavior (Ding et al., 2001). However, little is known about the toxic effect of AVM on birds.

The accumulation of AVM in the environment makes AVM a major candidate for toxicological studies. As a critical part of the digestive system and the general circulation system, liver plays an important role in metabolism and biotransformation of exogenous substances. AVM has been identified to be able to cause injury to liver cells (El-Shenawy, 2010; Maioli et al., 2012). Some other reports indicate that highly concentrated avermectins can induce apoptosis in isolated rat hepatocytes (El-Shenawy, 2010) and pigeon neuron (Wang et al., 2009). In previous studies, we carried out studies in vivo and in vitro studies. We found that AVM has cytotoxicity to brain neurons of King pigeon in vitro and the mechanism of neurotoxicity induced by AVM is closely related to apoptosis (Li et al., 2013a, 2013b). In vivo, the microscopic structures of the cerebrum, cerebellum and optic lobe altered obviously, the severity of which increased with the concentration of AVM and exposure time. Meanwhile, AVM could induce oxidative damage to the brain tissue and serum of pigeon (Li et al., 2013a, 2013b). Meanwhile, we also attentioned that the liver of the AVM-treated pigeons exhibited structural alterations, include swelling, part necrosis, and inflammatory cell infiltration. Furthermore, there were many small focal necrosis observed in liver (unpublished). The severity of which increased with AVM concentration. Based on the above experimental results we performed this study, in order to further determine the AVM toxicity mechanism to liver in pigeons.

Apoptosis can be induced by particular endogenous and exogenous factors (Mattson and chan, 2003). It is also involves apoptotic gene network. Caspase-3 has been implicated as a critical substance in initiating apoptosis (Nicholson and Thornberry, 1997). Caspase-8 could make mitochondrial release cytochrome C by cutting Bcl-2 interacting domain (BID). When it was bound by fas

Abbreviations: AVM, Avermectin; DNPH, 2,4-dinitrophenylhydrazine; DPC, DNA–protein crosslinks; EDTA, ethylenediamine tetraacetic acid; FasL, fas ligand; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; PCO, protein carbonyl; ROS, reactive oxygen species; SOD, superoxide dismutase

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http://dx.doi.org/10.1016/j.ecoenv.2013.09.021

ligand, fas can activate a signal transduction pathway that eventually resulted in apoptosis (Lasham et al., 2000; Nakamura et al., 2007). Yu et al. (2008) demonstrated that chlorpyrifos induced apoptosis in mouse retina via oxidative stress.

Oxidative stress, defined as a state of imbalance between the concentration of reactive oxygen species (ROS) and antioxidant defense mechanisms, contributes to the development of various pathologies in organisms (Cooke et al., 2006; Valko et al., 2006). The consequence of such an imbalance is the generator of oxidative damage to biomolecules (Pamplona and Costantini, 2011).Therefore, antioxidants are important in protecting organisms against oxidative damage from pathological conditions or exogenous damage (Costantini, 2008). Carbamate derivatives such as mancozeb have also been reported to be able to cause oxidative stress, DNA damage, and activation of the mitochondrial pathway of apoptosis (Calviello et al., 2006). Toxic heavy metals led to apoptosis, in which ROS are closely involved. Apoptosis and ROS were positively correlated with the exposure dose (Son et al., 2010, 2011).

According to OECD Guidelines for Testing of Chemicals, pigeon is a recommended species for toxicology experiments, since they are sensitive to the change of exogenous substances in the environment. Therefore, we choose pigeon as experimental animal for monitoring ecological environment pollution caused by AVM. Considering about possible injury cause by xenobiotics as mentioned above, we investigated the number of apoptosis, the expression of caspase-3, caspase-8, fas mRNA, ultrastructural characteristics, and antioxidant responses (SOD, GSH-Px, MDA), PCO content, DPC coefficient in pigeon liver that received AVM. These results provide valuable information for the possible mechanisms of apoptosis via oxidative stress exposing to AVM in the pigeon liver.

2. Materials and methods

2.1. Chemicals and materials

AVM: white crystalline 98% $AVMB_{1a}$ were purchased from the China Agricultural University Technology Development Corporation. Apoptosis detection kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). MDA assay kit (A003-1), GSH-Px assay kit (A005-1), and SOD assay kit (A001-1) were purchased from the Nanjing Jiancheng Biotechnology CO, Ltd (Nanjing, Jiangsu, China). All other reagents used in the analyses were of analytical grade and were obtained locally.

2.2. Animals and treatments

Eighty male and female American king pigeons, 2 months old (*Columba livia*) and weight of 300–350 g were selected. They were randomly allocated into 4 equal groups, and were fed with either a commercial diet (Control group) or a AVM-supplemented diet (20 mg/kg · diet (Low-dose group), 40 mg/kg · diet (Medium-dose group), or 60 mg/kg · diet (High-dose group) for 30, 60 or 90 days, respectively. Feed and tap water were supplied ad libitum. Following euthanasia with sodium pentobarbital, pigeon livers were quickly removed at the end of 30, 60, 90 days treatment. The remaining two pigeons in each group were standby for any unexpected condition. Liver tissues were collected and divided into three portions for situ apoptosis detection, ultrastructural observations and other biochemical indexes detection.

All pigeon experiments were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University under the approved protocol number SRM-06.

2.3. Ultrastructural observation

Samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 90 min, then rinsed three times in the buffer and left overnight. Later, the tissues were post-fixed for 2 h in 1% buffered OsO_4, dehydrated through ascending graded series of acetone, and infiltrated with Spurr's resin. After polymerization overnight at 70 °C, semi thin sections (0.5 mm) were cut with glass knives, stained with 1% toluidine blue O in 1% sodium borate and examined with a light microscope. Ultrathin sections (80–90 nm) were cut with a diatome diamond knife, collected on 200-mesh nickel grids, stained in 5% uranyl acetate for 10 min, and then counter-stained in lead citrate for 6 min. Sections were examined and photographed using a Jeol 100S transmission electron microscope.

2.4. Assessment of apoptosis

Immediately after being excised, the liver was fixed with 4% paraformaldehyde in PBS, dehydrated through ethanol and xylene, and embedded in paraffin. Briefly, 5 μm thick sections were cut on a Microtome (NO1026, Japan) and mounted onto poly-L-lysine-coated glass slides. TUNEL staining was performed using situ terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) biotin nick-end labeling (TUNEL) technique that identifies DNA strand breaks by labeling free 3′ –OH termini (In Situ Cell Death Detection Kit, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The result was calculated by averaging the number of apoptosis per microscopic field which was expressed as average percentages of apoptosis.

2.5. Determination of caspase-3, caspase-8 and fas genes expression

2.5.1. Total RNA isolation and reverse transcription

Total RNA was isolated from liver tissue samples using the TRLzol® reagent, according to the manufacturer's instructions (Invitrogen, America). The dried RNA pellets were resuspended in 80 μl of diethyl-pyrocarbonate-treated water. The concentration and purity of the total RNA were determined spectrophotometrically at 260/280 nm. First-strand cDNA was synthesized from 2 μg of total RNA using oligo (dT) primers and Superscript II reverse transcriptase according to the manufacturer's instructions (Takara, China). Synthesized cDNA was diluted 10 times with sterile water and stored at −80 °C.

2.5.2. Quantitative real-time PCR (qPCR)

Primer Premier Software (PREMIER Biosoft International, USA) was used to design specific primers for caspase-3, caspase-8, fas and ß-actin based on the known Gallus gallus sequences under the accession numbers L08165.1, NM204725, NM204592 and XM421659. The following set of primers were used for caspase-3: forward CACCACAGCCGAGAGAGAAAT; and reverse TGACCATCAGGGAGTTCATAGC; for caspase-8: forward CTGAAGGCTCCTGGTTTA; and reverse TGCCACTCTGCGATTTAC; for fas: forward GGAAGCGGGAAGATATTGAG; and reverse GCCCAGGTAGGAAGCTAGAA; ß-actin: forward GCACTCGGTTTGGAGGTTGT; and reverse CGTGGCATTCCTGCTTCTT. The amplification products were caspase-3, 135 bp; caspase-8, 104 bp; fas, 143 bp and ß-actin, 197 bp. qPCR was used to detect the expression of caspase-3, caspase-8 and fas genes in liver by using SYBR Premix Ex Taq (Takara, Shiga, Japan). Reaction mixtures were incubated in the ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The program was 1 cycle at 95 °C for 30 s and 40 cycles at 95 °C for 5 s and at 61 °C for 34 s. Dissociation curves were analyzed by Dissociation curve1.0 Software (Applied Biosystems) for each PCR reaction to detect and eliminate possible primer–dimer formation and nonspecific amplification. The magnitude of change in gene expression relative to controls was determined by the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001).

2.6. Detection of antioxidant enzyme activities

Liver tissues were homogenized in 5 mL ice-cold Tris–HCl buffer (0.01 mol/L, pH7.4) containing 0.01% ethylenediamine tetra-acetic acid (EDTA), 0.01 mol/L saccharose, and 0.8% NaCl. The homogenization procedure was carried out for 10 min at 654 g. All procedures were performed at 4 °C. Homogenate, supernatant, and extracted samples were prepared to determine antioxidant enzyme activities (MDA, SOD, and GSH-Px assay) which according to the manufacturer's instructions.

2.7. Determination of PCO level

PCO content was tested by 2,4-dinitrophenylhydrazine (DNPH) spectrophotometry with some modifications (Levine et al., 1990). Briefly, 100–200 mg tissue was thawed and homogenized at 10% (w/v) in HEPES buffer {pH 7.4, containing 10 mM HEPES, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.6 mM $MgSO_4$, 40 mg/LPMSF, 0.5 mg/L protease inhibitor, 0.7 mg/L pepsin inhibitor and 1.1 mM EDTA}, using glass homogenizer in ice. The supernatant was used for DNPH-reaction. PCO content was calculated by the absorbance at 370 nm using the extinction coefficient of 22,000 $M^{-1}cm^{-1}$ for aliphatic hydrazones, and the result was expressed as nmol carbonyl/mg protein.

2.8. Measurement of DPC coefficient

Sample was washed with ice-cold phosphate-buffered saline, and was forced through a wire-mesh screen to obtain 1.5×10^6 mL^{-1} single-cell suspension. The suspension was treated with sodium dodecyl sulfate (SDS, 2%) at 65 °C for 10 min, then KCl (1 M in 10 mM Tris–HCl, pH 7.4) was added followed by passing the